Effect of TERT on the growth of fibrosarcoma via caspase-3, survivin and PKB

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Abstract. The present study explored the effect of telomerase reverse transcriptase (TERT) on the growth and apoptosis of fibrosarcoma, and investigated the potential molecular signalling pathways underlying its effect. A plasmid was constructed in order to overexpress TERT and siRNA was used to knockdown TERT. The effect of TERT on fibrosarcoma cells *in vitro* was studied by performing reverse transcription-quantitative PCR and western blotting to determine the expression of p53, survivin, caspase-3, caspase-7 and PKB. Knockdown of TERT suppressed cell growth, decreased fibrosarcoma volume, decreased survivin and PKB expression, and increased caspase-3 expression. The results of the present study suggest that TERT regulates the growth of fibrosarcoma *in vitro* and *in vivo*, and that this is associated with the expression of caspase-3 and survivin, in addition to the PKB signalling pathway.

Introduction

Telomerase reverse transcriptase (TERT), known as hTERT in humans, is the catalytic component of telomerase, a type of nuclear reverse transcriptase responsible for telomere extension in cells (1). A previous study demonstrated that abnormal expression of hTERT leads to the progressive shortening of telomeres, which could induce cell immortalization and malignant tumor growth (2). The function of hTERT *in vivo* and *in vitro* was studied directly by cloning the open reading frame (ORF) of the target gene into expression vectors with different protein tags, a technique used in a previous gene function study (3).

In the current study, ORF expression cloning technology was used with the TERT gene as a target to design and construct

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a TERT-ORF clone vector and produce virus particles with the ability to express TERT through viral packaging. The effects of TERT on fibrosarcoma *in vitro* were studied.

Materials and methods

Cell lines and cell culture. Human fibrosarcoma HT1080 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in Minimal Essential Medium supplemented with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C with 5% CO_2. Lenti-Pac 293Ta cells (GeneCopoeia, Inc., Rockville, MY, USA) cultured using Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin in the same conditions. Human non-small cell lung carcinoma cell line H1299 cells (American Type Culture Collection) were cultured with RPMI-1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin, also in the same conditions.

Transduction of the Lv130 human immunodeficiency virus (HIV) lentiviral vector. A total of 10 ng of Lv130 HIV lentiviral vector (GeneCopoeia China Inc., Guangzhou, China) was added to 100 μl competent *Escherichia coli* cells (cat no., C7373-03; Invitrogen; Thermo Fisher Scientific, Inc.) and incubated on ice for 30 min, then immediately transferred to a 42˚C water bath and heat shocked for 60 sec, then transferred to ice for 2 min. Super optimal culture medium (200 μl; Sigma-Aldrich China, Beijing, China) was added and the sample was incubated at 37˚C with agitation for 1 h at 200 rev/min, then 100 μl was cultured on an LB plate with ampicillin at 37˚C overnight. The plasmid was isolated with a Plasmid Miniprep kit (cat. no., PFM250; Sigma-Aldrich China) according to the manufacturer's protocol. Restriction endonuclease digestion and 1% agarose gel electrophoresis with ethidium bromide were performed to confirm that the TERT sequence had been incorporated into the plasmid.

Construction of the recombinant plasmid with lentivirus vector and TERT gene DNA. The TERT gene was obtained by polymerase chain reaction (PCR) analysis. The total RNA of nude mice brain (supplied by Laboratory Animal Center of the

Harbin Medical University, Harbin, China) was isolated with RNeasy Plus Universal kits [cat. no. 73404; QIAGEN (Suzhou) Translational Medicine Co., Ltd., Suzhou, China] according to the manufacturer's protocol. Primers were designed according to the mouse TERT gene sequences in GenBank. Primer sequences for TERT were as follows: Forward, 5'-GCGGTA GGCGTGTACGGT-3' and reverse, 5'-CGATCTCGAACT CGTGGC-3'. Primers contained restriction sites for *Nhe*I and *Bsp*119I. Reverse transcription (RT)-PCR was performed with a One Step RT-PCR kit [cat. no. 210212; QIAGEN (Suzhou) Translational Medicine Co., Ltd.]. The RT-PCR product (TERT gene) and the lentiviral vector were digested with *Nhe*I and *Bsp*119I restriction endonucleases. The products of enzyme digestion were purified with a QIAquick Gel Extraction kit [cat. no. 28704; QIAGEN (Suzhou) Translational Medicine Co., Ltd.] and ligated with T4 DNA ligase to construct the recombinant plasmid.

Packaging of the virus. Cells at a confluence of 70-80% were used for transfection. The lentiviral packaging plasmid mixture (including 0.5 μg recombinant plasmid and 1 μl virus) was co-transfected into 293Ta cells and H1299 cells, which were incubated at room temperature for 25 min, then added into 6-well plates and cultured at 37°C with 5% CO_2. The medium containing transfection mixture residues was discarded and fresh medium was added following 12 h incubation. The cell supernatant was collected after 48 h by centrifugation (1,000 x g at 4°C for 5 min), then a 0.45 μm polyvinylidene fluoride (PVDF) film was used to filter and harvest the packaged virus particles.

Overexpression of TERT. The recombinant lentiviral overexpression vector was transfected into HT1080 cells using Lipofectamine 2000 (cat. no. 11668027; Thermo Fisher Scientific Inc.), according to the manufacturer's protocol.

RNA interference (RNAi) of TERT. HT1080 cells at confluence of 70-80% were used for RNAi. TERT-siRNA: AATCAGACAGCACTTGAAGAGGG was used for transfection of HT1080 cells using Lipofectamine 2000. Fluorescent images were captured following culture for 48 h to observe the growth status of the cells. Five images of each culture well were captured in the visual fields located in the center, upper, lower, left and right of the well.

RT-quantitative(q) PCR. Total RNA was extracted from cells subjected to the knockdown or overexpression of TERT after 72 h, using the RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. Total RNA (1 μg) was subjected to reverse transcription using a reverse transcription system (cat. no. A3500; Promega Corporation, Madison, WI, USA). qPCR was performed using SYBR-Green PCR Master Mix (Promega Corporation). Thermocycler conditions were as follows: 95°C for 45 sec; 95°C for 5 sec; and 60°C for 30 sec, for 40 cycles. Primers used were as follows: p53 forward, 5'-GCCATGGCCATCTACAAG-3' and reverse, 5'-CCTTCCACCCGGATAAGAT-3'; survivin forward, 5'-TTCAAGAACTGGCCCTTC-3' and reverse, 5'-CCTTAA AGCAGAAAAAACACTG-3'; caspase-3 forward, 5'-TTC TTCAGAGGCGACTACT-3' and reverse, 5'-TCCCACTGT
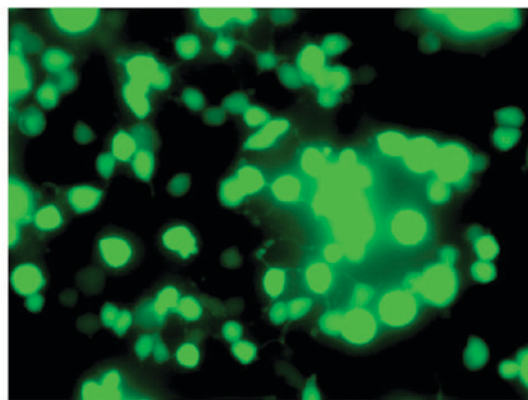

Figure 1. Fluorescence microscopy image of HT1080 cells following transfection with the human telomerase reverse transcriptase plasmid (magnification, x200).

CTGTCTCAAT-3'; and caspase-7 forward, 5'-TCTTTGCTT ACTCCACGGTT-3' and reverse, 5'-ACCCTGGTCAGGATC TGCAT-3'; GAPDH (internal control) forward, 5'-TGTGGG CATCAATGGATTTGG-3' and reverse, 5'-ACACCATGT ATTCCGGGTCAAT-3'. The results were quantified using the $2^{-\Delta\Delta Cq}$ method (4).

Detection of the expression of protein kinase B (PKB) with western blotting. Briefly, cells that had been subjected to the knockdown or overexpression of TERT were lysed 72 h after RNAi in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA and protease inhibitor at 4°C for 2 h. Total protein (50 μg) was separated by 15% SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with primary antibodies against PKB (dilution, 1:1,000; United States Biological, Salem, MA, USA) at 4°C overnight. The secondary antibodies were added at a dilution of 1:1,000, incubated at RT for 2 h and the bands were stained with DAB. They were quantified using β-actin (dilution, 1:1,000; antibody cat. no. 143128; United States Biological) as the control. The protein bands were quantified using Image J software (version k 1.45; National Institutes of Health, Bethesda, MA, USA; https://imagej.en.softonic.com/).

Statistical analysis. Statistical analysis was performed using the SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA) and a one-way analysis of variance was conducted for the comparison between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Successful construction of the recombinant plasmid with the lentivirus vector and TERT gene DNA. The recombinant plasmid was isolated and confirmed to possess the ORF of the TERT gene by enzymatic digestion with restriction endonucleases *Nhe*I and *Bsp*119I, and sequencing of the plasmid (data not shown). Green fluorescence was observed in viable HT1080 cells following transfection with the recombinant TERT plasmid (Fig. 1), confirming expression of the plasmid.
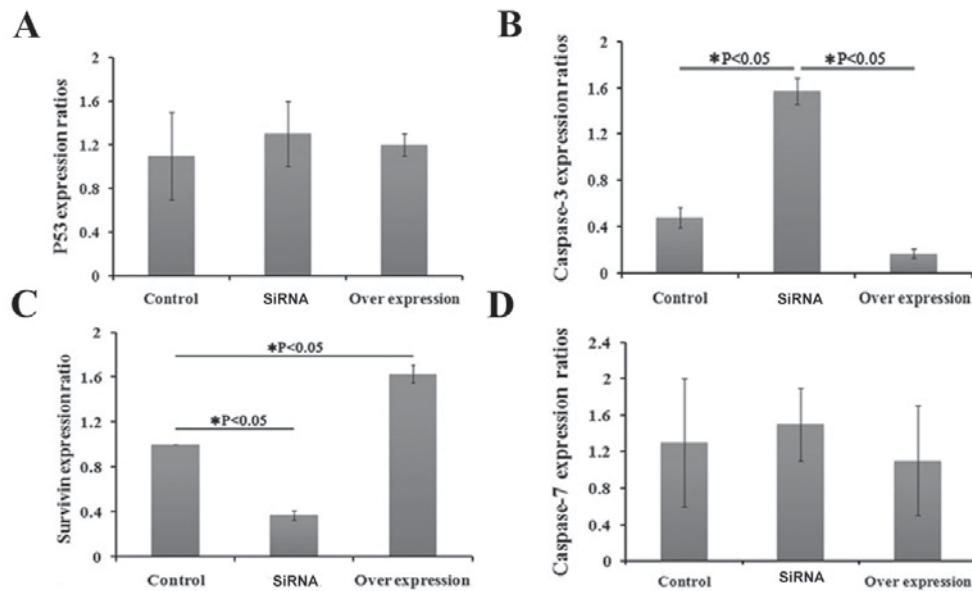
Figure 2. Reverse transcription-quantitative polymerase chain reaction analysis of the expression of (A) p53, (B) caspase-3, (C) survivin and (D) caspase-7. Error bars represent standard deviation. siRNA, small interfering RNA.
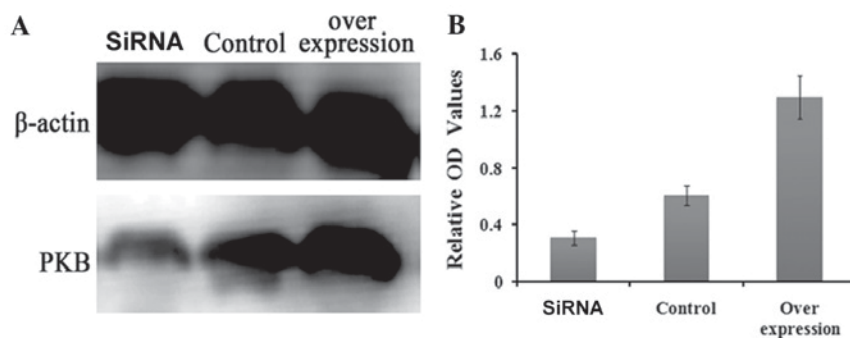

Figure 3. Western blotting results. (A) Western blot showing PKB expression in control, TERT siRNA-treated and TERT plasmid-treated HT1080 cells. (B) Quantification of PKB protein levels from western blotting. Error bars represent standard deviation. TERT, telomerase reverse transcriptase; PKB, protein kinase B; OD, optical density; siRNA, small interfering RNA.

Survivin and PKB expression decreases but caspase-3 expression increases following TERT knockdown. The effects of recombinant plasmid transfection on the expression levels of p53, survivin, caspase-3 and caspase-7 in HT1080 cells were detected by RT-qPCR. There was no significant difference in the expression levels of p53 and caspase-7 genes prior to and following TERT knockdown ($P>0.05$; Fig. 2). However, the expression levels of survivin decreased significantly and the expression levels of caspase-3 increased significantly following TERT knockdown ($P<0.05$; Fig. 2). The expression levels of survivin increased significantly and the expression levels of caspase-3 decreased significantly following TERT overexpression (Fig. 2).

Western blotting analysis revealed that the expression of PKB decreased significantly at the protein level following TERT knockdown ($P<0.05$), while it increased significantly when TERT was overexpressed ($P<0.05$; Fig. 3).

Discussion

The biological role of TERT is to add telomeric DNA to the end of eukaryotic chromosomal DNA to ensure the activity of cells (5). Telomeres serve an important role in maintaining cellular chromosome stability and activity in the cells of various species (6). In the present study, TERT overexpression significantly promoted the growth of HT1080 cells, while TERT knockdown significantly inhibited growth. TERT affects cell growth, as it regulates the expression of telomerase, which affects telomere length, and the length of telomeres can affect the cell growth state (7-9). Previous studies have demonstrated that TERT knockdown inhibits the growth of bladder cancer and prostate cancer (2,10). These results suggest that the TERT gene can control fibrosarcoma growth.

Previous studies have identified that TERT inhibits cell apoptosis (11,12), which could account for the increased tumor growth observed following TERT overexpression. In the current study, the expression levels of caspase-3, caspase-7, survivin and p53 were measured prior to and following TERT knockdown. TERT knockdown did not significantly affect the expression of caspase-7 or p53; however, it significantly affected the expression of caspase-3 and survivin. Survivin has been demonstrated to promote cell survival (13), whereas caspase-3 promotes apoptosis (14). In the present study, TERT

overexpression and knockdown significantly decreased and increased caspase-3 expression, respectively, indicating that TERT may serve a role in the growth of fibrosarcoma through caspase-3 and survivin.

PKB is a signalling molecule that regulates cell apoptosis and survival, and serves an important role in wound repair (15,16). In the present study, TERT was identified to regulate the expression of PKB. Previous studies have demonstrated that the PKB signaling pathway is widely associated with cell apoptosis, survival, growth and protein synthesis (17-20). Signal transmission in the cytoplasm through PKB promotes cell cycle progression by glycogen synthase kinase-3 β (21), and accelerates cell apoptosis through the inhibition of p21 or p53 (22). The results of the present study suggest that TERT affects the growth of fibrosarcoma via cell survival and apoptosis through the PKB signalling pathway.

The present study established a fibrosarcoma cell model overexpressing TERT. In this cell line, the effects of TERT on cell growth appeared to be mediated through the survivin, caspase-3 and PKB signalling pathways. These results provide important information for the application of hTERT-targeted therapies for the treatment of fibrosarcoma and similar malignancies, such as giant cell bone tumors.

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